Human amniotic fluid derived cells can competently substitute dermal fibroblasts in a tissue-engineered dermo-epidermal skin analog

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Abstract: PURPOSE: Human amniotic fluid comprises cells with high differentiation capacity, thus representing a potential cell source for skin tissue engineering. In this experimental study, we investigated the ability of human amniotic fluid derived cells to substitute dermal fibroblasts and support epidermis formation and stratification in a humanized animal model. METHODS: Dermo-epidermal skin grafts with either amniocytes or with fibroblasts in the dermis were compared in a rat model. Full-thickness skin wounds on the back of immuno-incompetent rats were covered with skin grafts with (1) amniocytes in the dermis, (2) fibroblasts in the dermis, or, (3) acellular dermis. Grafts were excised 7 and 21 days post transplantation. Histology and immunofluorescence were performed to investigate epidermis formation, stratification, and expression of established skin markers. RESULTS: The epidermis of skin grafts engineered with amniocytes showed near-normal anatomy, a continuous basal lamina, and a stratum corneum. Expression patterns for keratin 15, keratin 16, and Ki67 were similar to grafts with fibroblasts; keratin 1 expression was not yet fully established in all suprabasal cell layers, expression of keratin 19 was increased and not only restricted to the basal cell layer as seen in grafts with fibroblasts. In grafts with acellular dermis, keratinocytes did not survive. CONCLUSION: Dermo-epidermal skin grafts with amniocytes show near-normal physiological behavior suggesting that amniocytes substitute fibroblast function to support the essential cross-talk between mesenchyme and epithelia needed for epidermal stratification. This novel finding has considerable implications regarding tissue engineering.

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ORIGINAL ARTICLE

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Keywords Amniocytes · Amniotic fluid · Mesenchymal stem cells · Skin · Tissue engineering · Collagen hydrogel

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Introduction

The composition of the amniotic fluid changes with gestational age. Whereas in the first half of gestation most of the fluid comes from active sodium and chloride transport across the amniotic membrane and the fetal skin, most of the fluid in the second half of gestation results from fetal micturition and lung water production [1]. The cells found in this fluid stem from all three germ layers and stem from the fetal skin, the digestive tract, and from the amniotic membranes [2]. Among the heterogeneous population of fetal cells that can be found in the amniotic fluid are mesenchymal, hematopoietic, epithelial, and trophoblastic cells [3].

Previous studies showed that the mesenchymal cells from the amniotic fluid (in this study, referred to as

amniocytes) express a phenotypic profile that is consistent with the profile of fetal mesenchymal progenitor cells, i.e., they are negative for CD31 and positive for vimentin, α-smooth muscle actin, keratin 8 and 18, and fibroblast surface protein [4, 5]. They express several, but not all, important markers for embryonic stem cells, i.e., they are positive for Oct-4, Nanog, and SSEA-4, indicating that they represent a new class of stem cells, situated between embryonic stem cells and adult stem cells [3, 6]. It was shown that amniocytes have an expansion capacity four to eightfold higher than bone marrow derived mesenchymal stem cells, and that they differentiate into various mesenchymal lineages, including fibroblasts, osteocytes, and adipocytes [7]. In culture, these cells secrete various cytokines and chemokines important for wound healing, such as IL-6, IL-8, TGF-β, TNFRI, VEGF, and EGF [8]. Culturing of these cells was successful over a period of 8 months with stable karyotype [9]. In addition, it was shown that amniocytes retain their proliferative capacity and differentiation potential also during decades of cryopreservation [10].

Autologous fetal tissue engineering was performed in different animal models for various postnatal surgical interventions such as bladder augmentation, diaphragmatic replacement, tracheal augmentation, and skin defects [11–14]. Of note, in these models, tissue engineering was performed using a biopsy from the respective organ and the engineered tissue was delivered only after birth.

For fetal tissue engineering purposes, the amniotic fluid is a very attractive cell source. Cells can easily be harvested from amniotic fluid aspirated during the frequently performed diagnostic amniocenteses [5]. The goal of this paper was to test whether amniocytes could be used for tissue engineering of skin. In particular, we wanted to investigate whether these amniocytes finally can assume anatomically and functionally the role of dermal fibroblasts.

Materials and methods

Cell harvest and culture

Human skin samples were obtained after informed consent from patients and/or parents; all described studies were approved by the Medical Ethical Committee of the Kanton Zurich. Human primary fibroblasts and keratinocytes were isolated and cultured according to the standard protocol as previously described [15].

Confluent back-up human amniocentesis cultures were received after informed consent of the patients from the clinical cytogenetics laboratory of the University Medical Center Groningen. Cells were cultured from healthy fetuses (with a normal karyotype). Cells were harvested and expanded in DMEM (Lonza, Breda, The Netherlands) supplemented with 20 % fetal calf serum (FCS; Perbio Science, Ettenleur, The Netherlands) and 1 % penicillin, 1 % streptomycin (10,000 U/ml, Gibco, Paisley, UK), and 2 mM L-glutamine (Lonza, Breda, The Netherlands).

Engineering of dermo-epidermal skin substitutes

Skin grafts with either normal human dermal fibroblasts or human amniocytes in the dermis were prepared, using a previously established transwell system [6-well cell culture inserts with membranes of 3 μm pore-size (BD Falcon, Basel, Switzerland)] [15–17]. As negative control, skin substitutes with an acellular dermis were produced.

Briefly, bovine collagen type I (BD Biosciences, Basel, Switzerland) was mixed with either 1×10^5 human primary dermal fibroblasts or with 1×10^5 human amniocytes, or, for the negative control, no cells, and neutralized with a buffer containing NaOH [18]. The solution was poured into the cell culture inserts. The hydrogels were plastically compressed [19] and cultivated in DMEM with 10 % FCS. After 5 days, 7.5×10^5 keratinocytes were seeded onto the complete surface of all of the hydrogels. The grafts were cultivated for 3 days under submersed conditions in Rheinwald and Green keratinocyte medium, followed by 3 days cultivation at the air–liquid interface before transplantation onto immuno-incompetent rats. Medium was changed every 2–3 days.

Bright light microscopy

To compare the morphology of amniocytes and fibroblasts, bright light microscopic pictures were taken using a Nikon SMZ1500 stereo microscope with a Nikon DXM1200F camera.

Fluorescein diacetate live cell staining

The viability and morphological shape of the cells incorporated into the collagen type I hydrogels was compared using fluorescein diacetate (FdA) live cell staining [20]. Cell culture medium was removed and the hydrogels were incubated with 5 μM FdA (Sigma, Buchs, Switzerland) in PBS (Invitrogen, Basel, Switzerland). After 2 min, the FdA solution was removed and the hydrogels were washed with PBS. Fluorescein fluorescence was observed using a Nikon SMZ1500 fluorescent stereo microscope (FITC filter, Nikon DXM1200F camera).

Transplantation of dermo-epidermal skin grafts

Animal experiments were approved by the local committee for Experimental Animal Research. Immuno-incompetent

nu/nu rats (age 8–10 weeks, Harlan, Horst, The Netherlands) were prepared as previously described [16, 17, 21, 22]. To prevent wound closure by the surrounding skin, steel rings (diameter 26 mm) were implanted into full-thickness skin defects on the back of the rats and served as a modified Fusenig chamber [23]. The transplants were inserted into the ring and covered with a silicon foil (Silon-SES, BMS, Allentown, PA, USA). Animal numbers were: amniocyte-grafts excised 7 days post transplantation ($n = 6$); amniocyte-grafts excised 21 days post transplantation ($n = 6$); fibroblast-grafts excised 7 days post transplantation ($n = 6$); fibroblast-grafts excised 21 days post transplantation ($n = 6$); grafts with acellular dermal part excised 21 days post transplantation ($n = 6$). Wound dressing changes and photographic documentations (Nikon D90) were performed every 7 days. Animals were killed 7 or 21 days post transplantation. Transplants were excised, halved, and embedded in O.C.T compound (Tissue-Tek®, Sakura Finetek, Japan), or fixed in 4 % paraformaldehyde (Medite Medizintechnik AG, Nunningen, Switzerland) and embedded in paraffin (McCormick, Richmond, USA).

Histology and immunofluorescence

Paraffin sections (sectioned at 10 µm with a microtome from Leica, Wetzlar, Germany) were deparaffinized and stained with hematoxylin & eosin (Sigma, Buchs, Switzerland) and imaged by light microscopy (Nikon Eclipse TE2000-U inverted microscope connected with a DXM1200F digital camera (Nikon AG, Egg, Switzerland).

O.C.T-embedded tissue was frozen at −20 °C and sectioned at 10 µm (Leica, Wetzlar, Germany). Sections were permeabilized in ice-cold acetone for 5 min, air-dried, and washed three times with phosphate buffered saline (PBS, Invitrogen, Basel, Switzerland). Blocking was performed with PBS containing 2 % bovine serum albumin (Sigma, Buchs, Switzerland) for 30 min at room temperature. Sections were incubated with the pre-labeled antibodies for 1 h at room temperature. After three washing steps with PBS, nuclei were stained with 1 µg/ml Hoechst 33341 (Sigma, Buchs, Switzerland) in PBS for 5 min at room temperature. After two final washing steps with PBS, sections were mounted with Dako fluorescent mounting solution (Dako, Baar, Switzerland).

Antibodies

The following antibodies were used: K1 (clone LHK1, 1:200, Novus Biologicals, Littleton, USA); K15 (clone SMP190, 1:50, Spring Biosciences, Fermont, USA); K16 (clone LL025, 1:100, Chemicon International, Temecula, USA); K19 (clone RCK108, 1:100, Dako, Glostrup, Denmark); Ki67 (clone B56, 1:100, ABD Serotec, Dusseldorf, Germany); Lam5α3 (clone P3H9-2, 1:100, Santa Cruz, Labforce AG, Nunningen, Switzerland) [24].

For immunofluorescence stainings, primary antibodies were pre-labeled with either Alexa488 or Alexa555 conjugated polyclonal goat F(ab')2 fragments, according to the manufacturer's instructions (Zenon Mouse IgG Labelling Kit, Molecular Probes, Invitrogen, Basel, Switzerland).

Fluorescence microscopy

Immunofluorescence stainings were analyzed using a Nikon Eclipse TE2000-U inverted microscope, equipped with Hoechst, FITC and TRICT filter sets and connected with a DXM1200F digital camera (Nikon AG, Egg, Switzerland). Images were processed with Photoshop 7.0 (Adobe Systems Inc., Munich, Germany).

Results

Amniotic fluid derived cells

Mesenchymal cells from human amniotic fluid (amniocytes) were isolated, cultured and passaged several times. Upon 90–100 % confluence, the amniocytes (Fig. 1a) demonstrated a rather irregular arrangement as opposed to the one of human fibroblasts (Fig. 1b) that appeared to be more "streamlined". The amniocytes shared the spindle-shaped morphology with fibroblasts.

Amniocytes were incorporated into bovine collagen type I hydrogels and FdA staining was performed 3 days thereafter to assess the viability of the cells as well as their morphology in this 3D culture system. FdA staining revealed high cell viability with spindle-shaped, dendrite, or stellate morphology of the cells (Fig. 1c), similar to fibroblasts in collagen hydrogel (Fig. 1d).

Macroscopic appearance of epidermis development on transplanted grafts

At transplantation of the three types of skin substitutes (amniocytes in the dermis, fibroblasts in the dermis, or, acellular dermis) on the back of immuno-incompetent rats, no macroscopic difference between the three types of transplants could be observed (Fig. 2a–c). Seven days post transplantation, complete take of all grafts in all animals was observed (Fig. 2d–f). The presence or absence of an epidermis was difficult to asses macroscopically at this early stage, although the transplants with acellular dermis appeared to have a rather moist surface (Fig. 2f), usually indicating a missing or poorly developed epidermis. Twenty-one days post transplantation, grafts containing amniocytes or fibroblasts showed a clearly epithelialized surface and

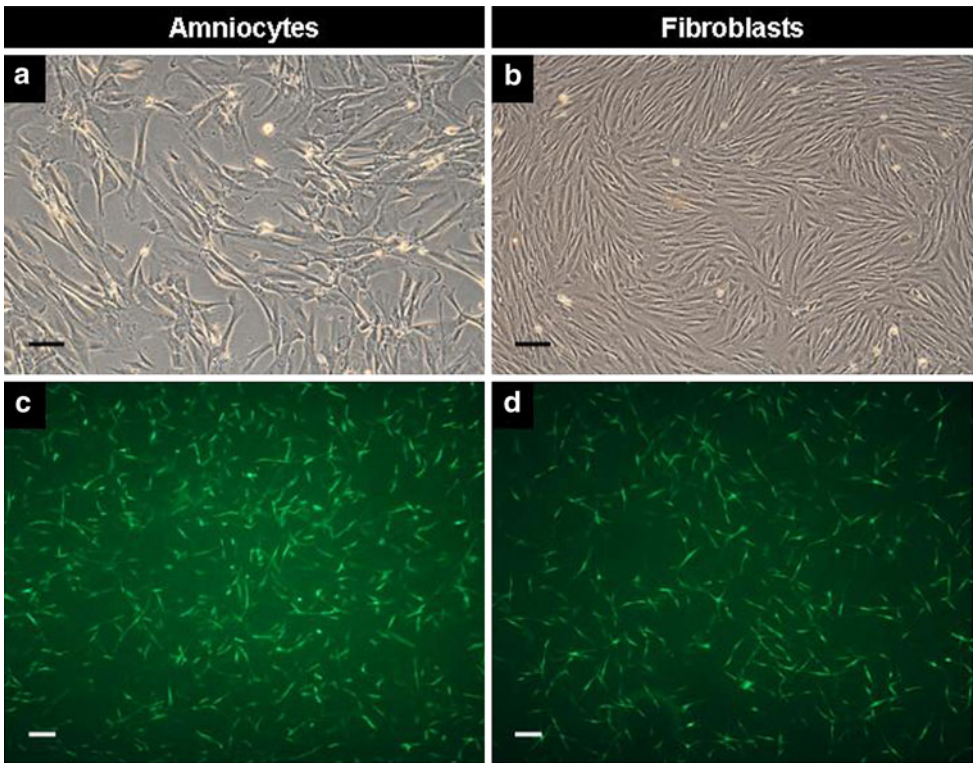

Fig. 1 Comparison of morphology of human primary amniocytes and human primary fibroblasts on cell culture plastic (2D) and in a collagen hydrogel (3D). **a** Phase contrast microscopy of amniocytes shows cell morphology similar to spindle-shape morphology of fibroblasts (**b**). **c** Positive fluorescein diacetate staining of amniocytes in collagen hydrogel shows dendrite morphology and good viability of the cells. **d** Fluorescein diacetate staining of fibroblasts indicating high viability as seen by the mainly dendrite or stellate morphology. *Scale bars* 40 μm

seemed well integrated into the rat tissue (Fig. 2g, h). Grafts with an acellular dermis had a moist surface (Fig. 2i) and appeared smaller compared to the other transplants.

Histological analysis of the epidermis

Excised grafts were sectioned and stained with hematoxylin & eosin. Seven days post transplantation, skin substitutes with amniocytes (Fig. 3a) showed a stratified epidermis of 4–6 keratinocyte layers with near-normal anatomy and a stratum corneum. The dermal part of these grafts was clearly distinguishable from the underlying tissue, and evenly distributed cells could be detected in the dermis (Fig. 3a). Similar features were observed in grafts with fibroblasts (Fig. 3b). Twenty-one days post transplantation, the neodermis in transplants with amniocytes and with fibroblasts showed a higher cell number than 7 days post transplantation, and the epidermis demonstrated about 6–8 layers on the amniocyte-grafts (Fig. 3c) and about 8–10 layers on the fibroblast-grafts (Fig. 3d). Twenty-one days post transplantation, a large part of the dermis in grafts with acellular dermis was devoid of any cells, and no epidermal elements were detectable (Fig. 3e).

Basal lamina deposition, epidermal homeostasis and proliferation

Grafts excised 21 days post transplantation were sectioned and immunofluorescence stainings were performed. Staining of the amniocyte-grafts with an antibody to the basal lamina component laminin 5 (Fig. 4a) demonstrated a deposition of a continuous basal lamina in all transplants and keratin 1 expression was not fully established in all suprabasal layers (Fig. 4a). In fibroblast-grafts in the dermis, laminin 5 staining revealed a continuous basal lamina and keratin 1 was expressed in all suprabasal layers (Fig. 4b). In amniocyte-grafts, keratin 16 was continuously expressed in all suprabasal layers (Fig. 4c). Amniocyte-grafts demonstrated more cells expressing the proliferation marker Ki67 in the basal layer (Fig. 4c), while the fibroblast-grafts demonstrated more Ki67 positive cells in the dermis (Fig. 4d). In fibroblast-grafts, keratin 16 expression was found in all suprabasal layers (Fig. 4d). In amniocytes-grafts, keratin 15 expression could be observed in most of the basal cells, whereas expression of keratin 19 was detected in most suprabasal cells (Fig. 4e). In fibroblast-grafts, keratin 15 expression was found in most basal cells, and a subpopulation of these basal cells was positive for keratin 19 (Fig. 4f).

Discussion

We describe here the generation of dermo-epidermal skin grafts containing human primary amniotic fluid derived cells in the dermis instead of human fibroblasts. These tissue-engineered skin substitutes were compared to tissue-engineered dermo-epidermal skin substitutes with human primary fibroblasts in the dermis and grafts with an

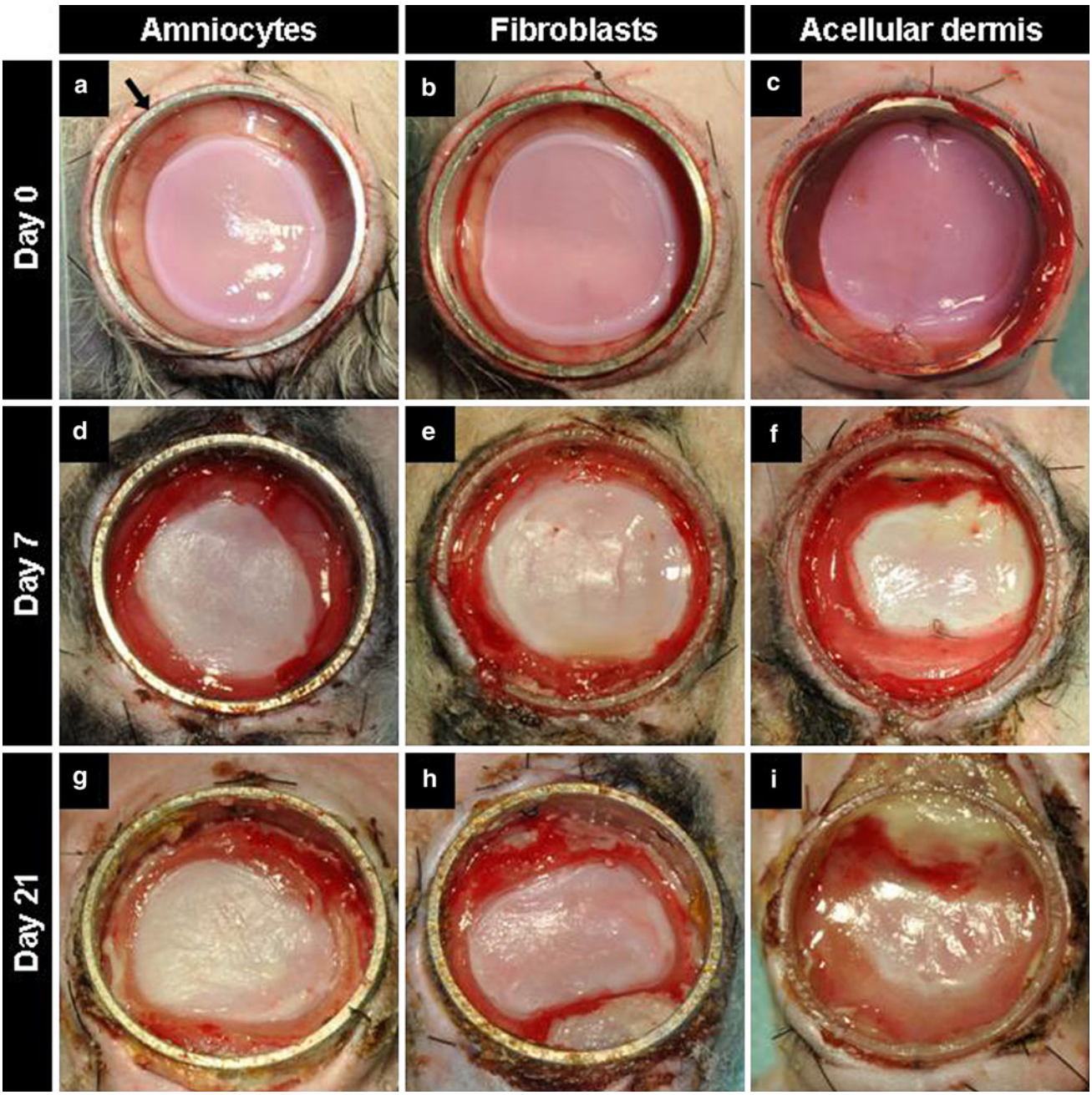

Fig. 2 Macroscopic views of grafts at time point of transplantation (day 0), 7 and 21 days post transplantation. **a**–**c** Transplants show similar appearance at the day of transplantation. **d**, **e** Seven days post transplantation, the surface of both amniocyte- and fibroblast-grafts is similar and looks rather dull and dry. **f** Grafts with acellular dermis appear more shiny and moist. **g**, **h** Twenty-one days post transplantation, grafts with amniocytes and with fibroblasts look similar as shown in **d**, **e**. **i** Twenty-one days post transplantation, grafts with acellular dermis look similar as described in **f**. Diameter of the ring (*arrow* in **a**) 26 mm (**a**–**i**)

acellular dermis. The key finding of our study is that the amniocytes can competently substitute for fibroblasts and successfully support epidermis stratification and survival. Some aspects deserve detailed consideration.

The interaction between epithelium and underlying mesenchyme was studied extensively for many years. It became clear that for successful epidermal stratification and survival, an underlying mesenchyme is absolutely mandatory. The mesenchyme provides essential growth factors to the epithelial cells and so stimulates the stratification of the epidermis [25–28].

It was therefore not surprising that we could not observe epidermis stratification, nor survival, in our skin grafts created with an acellular dermis. Interestingly, transplants

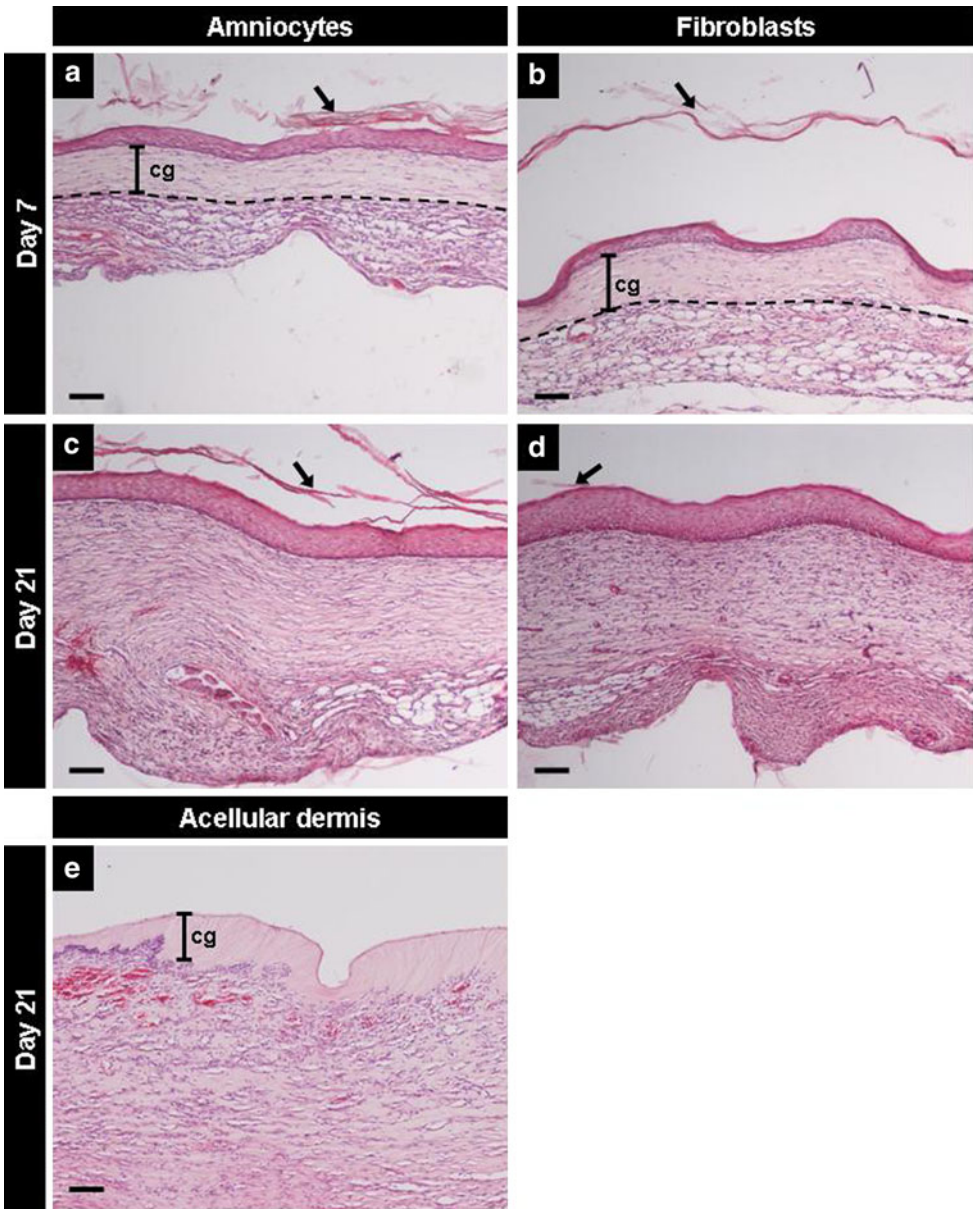

Fig. 3 Histological evaluation of the excised grafts (hematoxylin & eosin staining). **a** Seven days post transplantation, amniocyte-grafts show a correctly stratified epidermis with 4–6 cell layers and a stratum corneum. The collagen gel is easily discernable. **b** Seven days post transplantation, fibroblast-grafts look similar as described in **a**. The collagen gel is similar as in **a**. **c** Twenty-one days post transplantation, the epidermis of amniocyte-grafts is near physiological and has 6–8 cell layers. The dermis is markedly populated by cells. **d** Twenty-one days post transplantation, the epidermis of fibroblast-grafts is up to 8–10 cell layers thick including stratum corneum, and the dermis is densely populated by cells. **e** Twenty-one days post transplantation, grafts with acellular dermis show neither epidermis nor single epidermal cells. The collagen gel is easily detectable and a large part of it does not contain cells. The *dotted line* indicates the border between graft and underlying tissue. *Arrows* point at stratum corneum. *cg* Collagen gel. *Scale bars* 100 μm (**a**–**e**)

with amniocytes in the dermis showed near-normal structure, comparable to control transplants with fibroblasts in the dermis. The epithelial cells in amniocyte-grafts stratified to a near-normal epidermis including a stratum corneum and deposited a continuous basal lamina. However, expression patterns for markers of epidermal homeostasis suggested that 21 days post transplantation the epidermis was not yet in a fully homeostatic state. This aspect was reinforced by the detection of many suprabasal cells that were positive for keratin 19 expression, compared to the fibroblast-grafts where keratin 19 expression was restricted to some basal cells. These findings indicate that the development of the epidermis within amniocyte containing grafts is basically physiological, but slightly slower than in those containing fibroblast. These results provide compelling evidence that amniocytes incorporated into the dermal part of a dermo-epidermal skin graft can efficiently substitute fibroblasts and correctly support epidermal survival and stratification.

Diagnostic amniocentesis is routinely offered, can be safely performed under ultrasound guidance, and is associated with a very low rate of spontaneous abortion of only 0.5 % [29]. The removal of an additional small aliquot of amniotic fluid does not represent an additional risk to fetus or mother and also does not apparently constitute an ethical concern.

The present study can be seen as a first step toward tissue engineering of fetal skin without the necessity of harvesting a formal fetal skin biopsy. We demonstrate that prenatally harvested amniocytes can be successfully

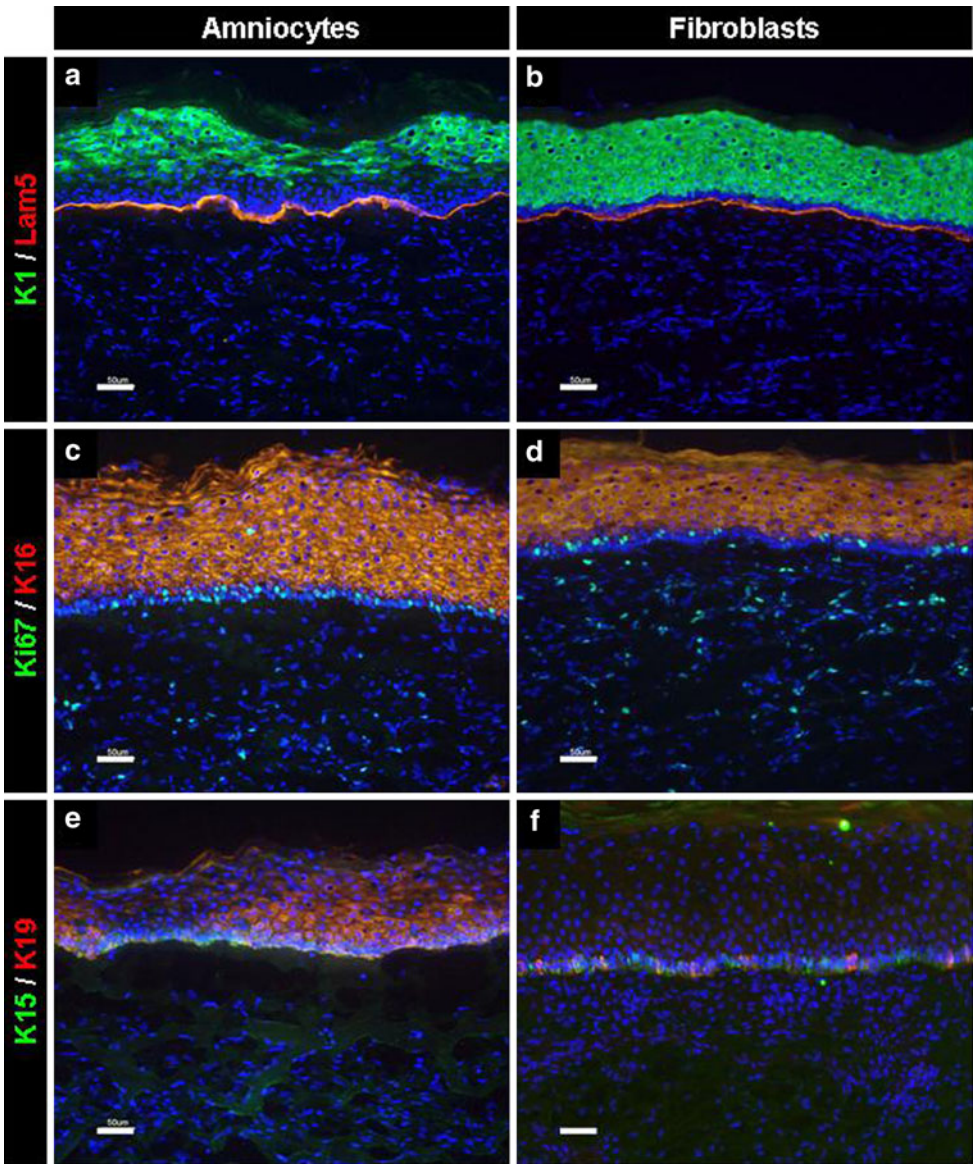

Fig. 4 Immunofluorescence stainings of grafts with amniocytes or with fibroblasts in the dermis 21 days after transplantation. **a**, **b** Staining of the basement membrane component laminin 5 (*red*) reveals the deposition of a continuous basement membrane in both types of transplants. Expression of keratin 1 (*green*) is not fully established in all suprabasal layers in amniocyte-grafts, whereas in fibroblast-grafts, all suprabasal layers express keratin 1. **c**, **d** In both types of transplants, keratin 16 (*red*) is expressed in all suprabasal layers. Proliferation as indicated by expression of Ki67 (*green*) in the basal cell layer of amniocyte-grafts is more pronounced compared to fibroblast-grafts. Proliferation of cells in the dermal part is lower in amniocyte-grafts than in fibroblast-grafts. **e**, **f** In amniocyte-grafts, keratin 19 (*red*) expression is detected in most suprabasal cells, whereas in fibroblast-grafts keratin 19 expression is restricted to some basal cells. In both types of grafts, keratin 15 expression (*green*) is detected in some basal cells. *Scale bars* 50 μm

employed to competently assume the role of dermal fibroblasts in a fetal-postnatal hybrid skin substitute (amniocytes = fetal cells, keratinocytes = postnatal cells). Of note, amniotic fluid also contains fetal epidermal cells that, hypothetically, might also be harvested, isolated, cultured, so as to form a fetal epidermis. Taken together, in a best case future scenario, autologous "fetal skingineering" could be accomplished by the sole use of amniotic fluid.

What are then, theoretically, possible applications for such laboratory-grown fetal skin? Human fetal surgery for spina bifida is a reality today and represents a novel standard of care for select fetuses suffering from that devastating malformation [30] (also at our center [31]). Quite often, the back lesions of these fetal patients are very big for successful primary skin closure. In those situations, an "off-the-shelf" autologous fetal skin substitute might represent an ideal way to guarantee adequate skin coverage. Another hypothetical application might be the use of amniotic fluid derived bioengineered fetal skin to treat chronic skin wounds, particularly in polymorbid elderly patients which often demonstrate notorious wound healing problems. It is in fact conceivable that fetal skin (even if allogenic) harbors more wound healing power [8] than postnatal skin substitutes used for the same purpose [32, 33]. Finally, one could even think of harvesting and cryopreserving amniotic fluid for any potential use later in life. It was in fact shown that stored amniocytes remain viable and functional over decades of cryopreservation [10]. If the same holds true for amniotic fluid derived epidermal cells,

then autologous skin might be engineered at any age of the patient for a number of possible indications (giant nevi, burns, scar revisions, chronic wounds, and other conditions where larger areas of skin are lost).

In summary and conclusion, we demonstrate here that amniocytes can competently assume the role of dermal fibroblasts in the context of bioengineering a full-thickness skin analog. This novel finding has fundamental implications on the emerging field of tissue engineering and harbors considerable potential regarding in vitro fabrication of both fetal and adult skin.

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Conflict of interest The authors declare that they have no conflict of interest.

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